Acta Medica 2014; 3: 51-54

acta medica

REVIEW

The Past, Present and Future of Gene Correction Therapy

Duygu AKÇAY[1], [BS],
Çetin KOCAEFE[1]*, [MD], [PhD]

ABSTRACT

The discovery of the structure of DNA and, consequently, of gene sequences accelerated efforts towards the implementation of genome technologies for the advancement of human health. Despite remarkable progress in biotechnology, human genetic diseases are still untreatable at present. Advancement in gene transfer technologies in the 90s initiated the first gene replacement therapy trials aiming at the correction of selected congenital genetic defects. By the beginning of the new millennium, progress in viral vectors primed the first successful clinical gene therapy initiatives. Today, biomedicine is on the edge of a new era with the implementation of genome editing technologies. The introduction of these novel techniques, gene correction therapy, which was no more than a fantasy in the last century, is now undergoing clinical trials. The key milestones of the nucleic acid technologies that laid the fundamentals of gene transfer and gene replacement therapy are summarized in this review with special emphasis on the three cardinal genome editing technologies: the Zinc-Finger Nucleases, TALE Nucleases and most recently the CRISPS/Cas system that made genome engineering possible. These novel applications recently initiated a wide range of uses, such as the generation of engineered cell lines, isogenetic model organisms and human clinical trials, towards gene correction aiming for the treatment of cancer, infectious diseases and single gene disorders. The current results and status of these novel approaches show great promise for development into therapeutic applications for the advancement of human health. This review is primarily directed at today's clinician-scientist as well as scholars and researchers in biomedicine.

Key words: gene therapy, single gene disorders, rare diseases, crispr/cas9, genome engineering

1 Hacettepe University Faculty of Medicine, Department of Medical Biology, Ankara

* *Corresponding Author: Çetin Kocaefe MD, PhD. Hacettepe University, Faculty of Medicine Department of Medical Biology, Ankara, Turkey*

e-mail kocaefe@hacettepe.edu.tr

Received 11August 2014, accepted 18 August 2014, published online September 2014

Seventy years ago, Avery, MacLeod and McCarty showed that through the transfer of DNA, unencapsulated "Rough" variants of Pneumococcus Type II could be converted into fully encapsulated cells [1]. Even though this ground-breaking study initiated the era of gene transfer and microbial genetics, it was initially undervalued by the scientific community. Following the unveiling of the structure of DNA, the genetic code and the concept structure of "the gene" were identified [2]. The next challenge was to understand the function of the genes and DNA sequences. Thus, the initial aim of the introduction of various DNA fragments into organisms was exploration of DNA function. These efforts led to the new era of recombinant DNA technology, where cutting and joining various DNA fragments could be used for the production of recombinant genes and proteins. This motive led researchers to seek various gene transfer approaches to target cells and organisms and fit various needs. Among these, biotechnology, the science of producing protein products, advanced much faster than other applications in biomedicine. The major challenge was to combine this new technology with efficient gene transfer techniques to express protein products in various ways. Among these, the cloning of the human growth hormone and insulin pioneered the expansion in biotechnology in the early 80s. The development of methodologies to produce recombinant proteins and enzymes for the advancement of human health represents key achievements in the history of medicine. Today, biotechnology for the advancement of

human health is capable of offering pure antigens for vaccination, human insulin molecule for the Type I diabetic patients as well as recombinant enzymes for lysosomal storage disease patients. While biotechnology applications are beyond the scope of this review, the number of recombinant protein products on the market as well as their indications increase each year. However, no FDA-approved gene therapy is currently available.

Initial efforts to identify disease-causing mutations and related genes date back to before 1980. At that time, this identification could only be achieved by using biochemical analysis of the protein products. Identification of the hemophilia A, Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Glucose-6-phosphate dehydrogenase (G6PD) genes were the initial successes of forward genetics [3]. The identification of the landmarks of the human genome and publication of the first human genetic linkage map accelerated the positional cloning of the genes [4, 5]. The completion of the human genome project in 2004 aided understanding of the gene function and establishing associations between mutations and disease mechanisms [6].

The discovery of the human genome sequence created anticipation for the implementation of this information on human health. However, crude DNA sequence information was just the end of the beginning of the struggle against genetic disorders. Currently, three fundamental therapy approaches exist in dealing with a disease caused by gene mutations. The first is known as "gene replacement therapy". Here, a "new" (i.e. artificial) DNA sequence is integrated into the host genome to compensate for the loss of the mutated protein product. This may be either achieved directly in vivo, or through re-administration of the genetically modified "autologous" cells known as ex vivo gene delivery. The classical clinical application of this approach was the much-debated "cure" for the X-linked severe combined immune deficiency (SCID-X1); the first gene therapy trial, in which eight boys had been cured [7]. The second approach is the modulation of the mutated gene structure to achieve a "partially functional" protein product. Skipping of the exon (s) that contains a mutation can be beneficial especially if the skipped exon would not disrupt the open reading frame. "Exon-skipping" is the alteration of the splicing mechanism to "skip" the mutated exons in order to transcribe a shorter mRNA with significantly higher function. Several obstacles and issues complicate putative gene replacement therapy for the muscle tissue, such as the size of the gene, successful targeting and delivery into muscle. Exon-skipping is one promising gene therapy approach for Duchenne's muscular dystrophy. Currently clinical trials using exon-skipping approach are ongoing [8]. Lastly, the "ultimate treatment" of a genetic disease is gene correction therapy. This approach can be briefly summarized as the direct in situ correction of the mutated gene. This process was considered to be no more than a fantasy in the last century. However, with the implementation of new tools, the promising approach of gene correction therapy will soon initiate a new age for the treatment of genetic disorders.

Gene Transfer

Initial efforts to transfer DNA into mammalian cells were an adaptation of prokaryotic transformation approaches. The priming protocols commonly precipitated DNA onto the in vitro cultivated mammalian cells. These protocols need to be precisely optimized for the cell type and were unsuitable for in vivo applications due to the lack of any options for targeting or tropism. Calcium phosphate precipitation (as exemplified in the next section) or formulation of DNA complexes with cationic compounds such as poly-ethylene imin (PEI) were partially replaced by liposomal structures over time. These non-selective chemical gene transfer approaches are still in practice today for research applications. In the past, a limited number of clinical gene therapy trials were also initiated using these direct gene transfer vectors [9]. Apart from the above-mentioned chemical complexes, there are also physical methods of gene transfer. Electroporation is one particular physical gene transfer technique in which an electric field is applied to drive DNA into selected cell types. This approach also has a special in vivo application niche in skeletal muscle, which is physically more resistant to such stress [10].

Following the introduction of viruses as efficient tools for gene transfer, the golden age of viral vectors was initiated. Adenoviruses, adeno-associated viruses, herpes simplex viruses and retroviruses were extensively studied with the goal of gene delivery into human cells. The first attempted gene replacement therapy using viral vectors was the debated trial of Martine Cline in 1990. His goal was to accomplish ex vivo gene replacement therapy to correct beta thalassemia using herpes simplex vectors

harboring the beta globin gene. Much controversy was created due to the fact that he did not obtain any institutional approval for this procedure to be applied on two patients. Despite the fact that none were harmed, neither was any success achieved [11]. Until the domestication of the lentiviruses, the majority of the past gene therapy approaches and clinical trials focused on Maloney Murine Leukemia Virus as a retroviral vector. The major breakthrough of domestication of the human immunodefiency virus (HIV) initiated the implementation of lentiviruses as stable gene therapy vectors [12].

A Brief History of Gene Replacement Therapy

The first gene transfer into mammalian cells was in 1962, in which Szybalska and Szybalski showed that mutant HPRT (-) bone marrow cell lines could be biochemically converted into an HPRT (+) state by calcium phosphate-mediated DNA transfer [13]. The authors not only described the methodology for in vitro gene transfer, but also laid the fundamentals of gene replacement therapy for the Lesch–Nyhan syndrome. In this rare genetic disorder, patients carry mutations in their HPRT gene [14]. Unlike the prokaryotic cells, even under optimal conditions, transfection efficiency of DNA into mammalian cells is very low. Besides several previous examples in the bacteria, Temin demonstrated that the Rous sarcoma virus was capable of introducing virus-specific genes into chicken cells and could induce a clear-cut phenotypic effect [15]. This provided proof that viruses could be used as tools to transfer genes into eukaryotic cells. The first in vitro demonstration of virus-mediated gene transfer was in 1973. Researchers inoculated the Shope virus in the tissue cultures of patient fibroblasts to restore arginase enzyme deficiency [16]. However, a clinical trial on hyperargininemic patients in which the virus was injected intravenously proved to be ineffective [17]. The first FDA-approved clinical trial was initiated in 1990 by William French Anderson to treat a genetic defect causing adenosine deaminase deficiency. Two children received ex vivo modified autologous white blood cells expressing the adenosine deaminase gene. Ashanti DeSilva, renowned patient and recipient of this approach, initially benefitted out of this trial but the impact proved to be temporary [18]. Naked DNA (plasmid) was also considered for the role of a gene transfer vector targeting skeletal muscle in myopathy patients. Plasmid DNA coding the dystrophin gene was delivered into the muscles of nine myopathy patients, but no beneficial effect was observed [19]. As mentioned above, the first sustainable successful clinical trial for a genetic disease was the gene therapy approach targeting the SCID-X1 gene. However, four of the nine boys who had enrolled in the trial developed acute lymphoblastic leukemia secondary to the insertional activation of a proto-oncogene, and one of them died [7]. Unfortunately, this occurred just after the tragic death of 18-year-old patient Jesse Gelsinger at the University of Pennsylvania in Philadelphia during another gene therapy trial [20]. These two incidents created a major setback for all gene therapy trials (with the resulting cessation of all similar trials) and also placed a black label on viral vectors.

Currently, the number of on-going gene therapy clinical trials exceeds 2000. Approximately 65% of these are cancer gene therapy trials. This is followed by the monogenic (9%), infectious (8%) and cardiovascular diseases (7%) [21]. About 65% of these trials are conducted in the U.S.A., followed by 10% in the UK, 4% in Germany, 2.5% in France and 2.4% in Switzerland. A dedicated website holds and updates the relevant catalogue information regarding the ongoing gene therapy trials as well as the past ones (www.wiley.co.uk/genemed/clinical). Successful gene therapy trials initiated for genetic diseases in the last decade include Leber's congenital amaurosis [22], β-thalassemia [23], ADA-SCID [24] and Wiskott-Aldrich syndrome [25]. Currently, Glybera® is the sole commercial gene therapy product on the market. Glybera® is a recombinant adeno-associated viral vector harboring lipoprotein lipase (LPL) gene and is approved to be used for LPL deficiency by the European Medicines Agency (EMA). Phase II clinical trial conducted in Europe proved efficient recovery from LPL deficiency by intramuscular injection of viral vectors [26]. However, FDA is still evaluating the approval mainly due to the immunological reactions raised against the capsid protein of the adeno-associated viral particles [27].

Gene Correction Therapy: The New Hope for Single Gene Disorders

Two principal routes are commonly pursued in the investigation of a gene function. The first one is known as "overexpression," in which the forced expression of the gene of interest is achieved in target cells (or transgenic animals). The second approach is the achievement of the "loss-of–function" that

would serve as a model for the human genetic disease caused by mutations. Both of these approaches enable the investigation of the ways in which genotypes may influence the phenotype [28]. The former can be modelled by direct gene transfer using appropriate vectors, as discussed above. Similarly, the transgenic animals can be generated by the stable germ-line integration of the transgene. The latter approach can only be accomplished by targeted mutations in the genome. The key to the implementation of targeted mutations is "homologous recombination" (HR) [29]. Through the implementation of this method, targeted sequence modifications as well as null mutations can be created in desired genes. Basically, HR is the strand exchange between identical DNA sequences [30]. Thus, an artificial DNA molecule (donor vector) with an identical sequence may trigger HR in the target locus with sequence similarity (Figure 1). Under physiologic conditions, this is an extremely rare event. Even in the presence of highly identical sequences, mammalian genomic DNA is extremely stable and spontaneous recombination practically never occurs. In the physiological state, homologous recombination frequency is estimated to be one in every 10^4 to 10^7 cells [28]. However, mammalian cells may use homologous recombination to repair DNA damage [31]. The below-described genome editing tools provide an opportunity to facilitate this recombination event.

The key to increasing the efficiency of homologous recombination is to follow the non-physiological route. Once a double strand break occurs, the probability of repair by homologous recombination is boosted [28]. The recent applications developed in the last fifteen years, termed as "genome editing tools," enable the achievement of targeted modifications in any gene, both in vitro and in vivo. These approaches are based on the creation of a precise double-strand DNA break (DSBs) in a target sequence (see the glossary box in table 1). This is accomplished through a "nuclease" function that would hydrolyze the phosphodiester bonds on both strands of a DNA helix. As explained above, such double strand breaks evoke DNA damage response and trigger HR. Here, the most important challenge is to direct this nuclease action specifically to the target DNA sequence. This sequence specificity can be achieved by engineered nucleases based on an enhanced DNA recognition property. This way, efficient and accurate genetic modifications can be achieved at a specific locus. Firstly, DSBs are introduced at the target locus, and then cellular DNA repair mechanisms are provoked to do the repair. The choice of the repair method

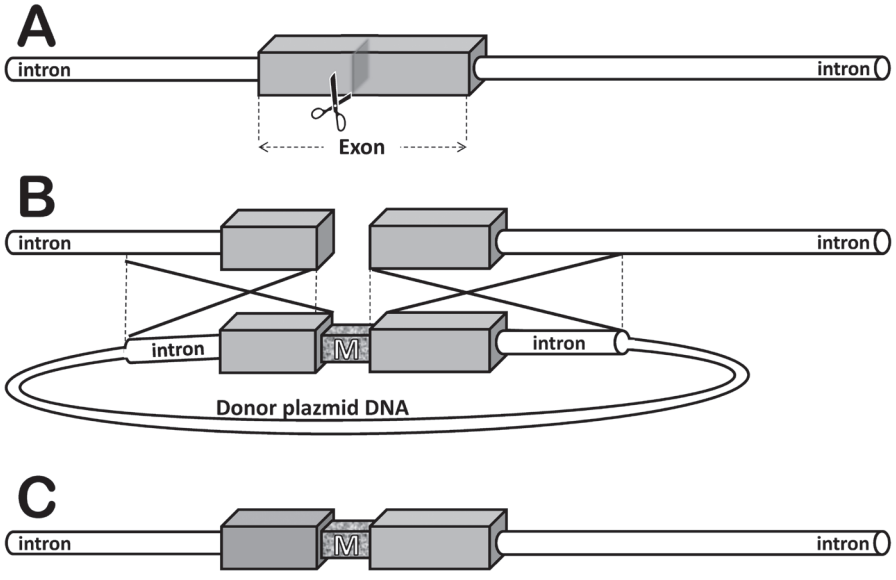

Figure 1. Principle mechanism of homologous recombination (HR) is illustrated. Any double-strand breakage (DSB) initiates the DNA repair mechanisms in the cell (A). HR mediated repair is initiated in the presence of an identical "homologous" sequence in the cell. Genome engineering approaches require a targeted nuclease to cleave the desired locus (to achieve the DSB) and an artificial DNA sequence to be delivered as a donor plasmid (B). HR mediated strand exchange incorporates the preferred nucleotide change (designated as M) existing in the donor plasmid vector into the genome (C).

Table 1.

Glossary box:

Double strand breaks (DSBs): The breakage of the backbone of a DNA chain that encompasses both strands. DSBs is DNA damage that occurs following exposure to ionization or ultraviolet radiation.

Isogenetic disease model: A model organism carrying the same disease causing mutation as in humans.

Nuclease: Enzymatic activity that hydrolyses the sugar-phosphate backbone of the nucleic acids, forming a DSB.

Restriction endonuclease: An enzyme that cleaves the sugar-phosphate backbone of the DNA by recognizing a specific sequence.

Zinc-Finger Proteins: Transcription factor with a certain structure (containing a zinc molecule) that exhibits sequence-specific DNA-binding activity.

defines the nature of the genome editing approach. Either a more error-prone "non-homologous end joining" (NHEJ) or HR is activated. The former, NHEJ, rapidly joins the broken ends. This mechanism exclusively introduces one or more base deletions or insertions at the site of repair. This process can be utilized to introduce loss-of-function mutations in desired genes. In case there is a donor DNA sequence available for homologous recombination, recombinational repair mechanism (HR) is then activated. Any desired sequence variation can be introduced using this engineered HR approach. The use of the targeted nucleases with or without recombination vectors is called "genome editing". Today, genome editing tools can be used to generate targeted mutations, gene replacement and gene correction, both in vitro and in vivo.

In order to gain sequence specificity to the nuclease action, site-specific DNA-binding proteins are utilized. These proteins are derived from (1) zinc-finger proteins (ZFPs) (2) transcription activator-like effector (TALE) proteins and (3) clustered regulatory interspaced short palindromic repeats (CRISPR/Cas) [30]. These three novel technologies are effectively used in many cell types and organisms and accelerate genome engineering studies. These novel tools also harbor promising clinical potential in gene replacement therapy. More than two decades have passed since FDA approved the first gene therapy trial, and the introduction of the genome editing technologies, gene therapy approaches have since accelerated significantly [32]. While ZFNs are already being tested in clinical trials, TALEN and CRISPR/Cas technologies are relatively new and have not yet been tested in any clinical applications. Nevertheless, they are currently widely in use for a range of cell lines and model organisms. The current results and the status of these new approaches show great promise to develop into therapeutic applications.

Zinc-Finger Nucleases

The first zinc-finger nucleases (ZFNs) were generated based on the DNA sequence recognition function of the zinc-finger transcription factors. Like all transcription factors, zinc-finger proteins possess a sequence-specific DNA-binding activity. An initial proof-of-concept study in 1994 was aimed at the generation of a chimeric protein by hybridizing the zinc-finger domain with a nuclease to enable generation of sequence specific DSBs [33]. Since then, the basic structural concept of ZFNs is laid on two separate domains, the DNA-binding and the DNA cleavage domains (Figure 2). The cleavage domain is primarily a nuclease without any specificity for a target sequence. Directing this domain to the specific locus is the function of the DNA-binding domain, or the zinc-finger domain. The DNA-binding domain of a ZFN contains Cys2His2 zinc-finger structures (ZFs) in each unit. Here, an atomic zinc molecule is surrounded by approximately thirty amino acids. A single zinc-finger typically recognizes 3 bp of DNA. The DNA-binding domains of ZFNs consists of three to six individual zinc-finger repeats enabling 9 to 18 bp DNA sequences to be identified. Thus, zinc-finger domains can be engineered to target specific sequences up to 18 bp. This is generally accepted to be sufficient to target a single locus in a mammalian genome. The nuclease domain of the ZFNs is generally derived from the Type II restriction endonuclease FokI. Type II restriction endonucleases are only active in a dimerized conformation. Thus, to achieve functionality, two identical domains need to dimerize around one target DNA. Considering the chimeric structure of an engineered ZFN, two separate zinc-finger domains need to recognize, and bind two opposite DNA strands of the target site for the two cleavage domains to surround and successfully dimerize around the DNA helix (Figure 2). This structure requires that the functionality to be reassured only

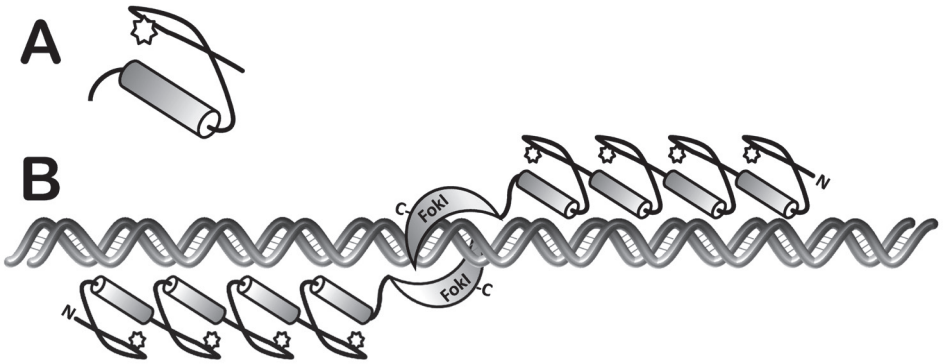

Figure 2. Basic mechanism of action of ZFNs is illustrated. ZFNs are made up of various tandem zinc finger (ZF) motifs (A). Each motif consists of an alpha helix (designated as the cylinder) as well as two beta sheets where an atomic zinc molecule is incorporated (designated as a star). One ZF motif can recognize and bind to 3 consecutive nucleotides on the single strand of a DNA helix. Structurally, a functional ZFN is a pair of tandem ZFNs targeting the two opposite strands of a DNA locus. Successive binding of the ZFNs on the two strands enables the functional pairing of the FokI nuclease to create a DSB (B). The optimization of the length of the linker sequence between the FokI nuclease domain and the tandem ZF motives is critical.

by the specific binding of the two separate ZF domains recognizing 18 bases, each of which are separated by 5 to 6 bp spacers. The two 18 bp inverted target recognition sequences reassure the specificity of this application [34].

ZFNs have been extensively used for engineering applications in the genomes of many plants and animals, including tobacco, flies, worms, zebrafish, mice, rats and numerous mammalian cell lines. Like other genome editing tools, ZFNs were also used for the generation of genetic disease models also known as "isogenetic human disease models" (see the glossary box in table 1) [35]. ZFNs were also evaluated as a potential treatment option for the HIV infection. The HIV virus requires the expression of co-receptors C-C chemokine receptor type 5 (CCR5) for adhesion onto the T-cells. Thus, CCR5 is a promising target for the control of HIV entry into the host cell. Humans who are homozygous for a particular variation in the CCR5 gene are naturally resistant to the HIV infection by the blockade of entry of the virus into the T-cells (the Δ32 variation). Additionally, in clinical use the transplantation of ex vivo expanded CCR5 (-/-) primary human CD4+ T-cells to HIV patients was found to be beneficial. In the light of this evidence, ZFNs were also tested as a potential option for the treatment of HIV infection. The primary aim of this was to disrupt the CCR5 in both primary CD4+ human T-cells and human hematopoietic stem cells. A Phase 1 clinical trial evaluating this approach was recently completed [36, 37]. Two separate Phase 1 clinical trials are still ongoing, in which T-cells are isolated from patients, treated with ZFNs to block CCR5 expression to provide an HIV-resistant reservoir of CD4+ T-cells and re-infused into the patients (NCT01044654 and NCT00842634) [31]. Once the targeted efficacy can be achieved, a potential treatment option for AIDS patients may be provided by ex vivo modification of their hematopoietic stem cells using CCR5-specific ZFNs. Reconstitution of the patients' immune systems using stem cells with an engineered "CCR5-negative genome" may render their T-cells immune to HIV infections.

TALE Nucleases

TALE nucleases are principally derived from the Xanthomonas bacteria, which are plant pathogens. Xanthomonas use a cocktail of thirty to forty different effector proteins during the infection of the host to damage eukaryotic cellular defense pathways [38]. The acronym TALE stands for Transcription Activator-Like (TAL) Effector and describes a family of proteins that can bind to specific genes in the plant genome to regulate their expression [39]. TALs contain 33 to 35 repetitive amino acid residues in a central domain, and the amino acid sequences of each repeat are almost identical. However, two contiguous amino acids at the 12th and 13th residues in the repetitive region serve a particular function in DNA-binding specificity [40]. These two hypervariable amino acids, known as the repeat-variable di-residues (RVDs), determine the specificity of the TALE proteins [32]. The cracking of the RVD code

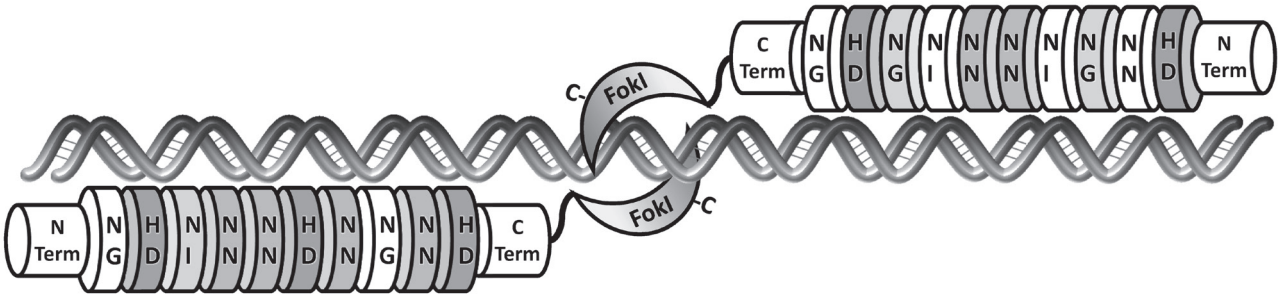

Figure 3. TAL-Like Effector Nucleases (TALENs) are targeted to a specific DNA sequence with the help of their repeat-variable di-residues (RVDs). Here, the consecutive pair of aminoacids shows specificity for corresponding nucleotides. These are NI (Asn, Ile) for adenine, HD (His, Asp) for cytosine, NN (Asn, Asn) or NK (Asn, Lys) for guanine and NG (Asn, Gly) for thymine. Each one of the two engineered TALE domains recognizes 18 – 20 bases on opposite strands of a DNA helix. This targeted binding functionally provides a specific conformation to the chimeric FokI nuclease domain that creates a specific DSB at the target locus. Optimization of the linker sequence between the FokI nuclease and the TALE motives is critical.

enabled the generation of engineered TAL effector proteins that may target any desired sequence in the genome and provide specificity to the FokI restriction enzyme to create a DSB at the target locus [41]. The DNA binding specificity of the RVD residues are coded by specific aminoacids that correspond to A, C, G and T nucleotides. These are NI (Asn, Ile) for adenine, HD (His, Asp) for cytosine, NN (Asn, Asn) or NK (Asn, Lys) for guanine and NG (Asn, Gly) for thymine. Like the ZFNs, for genome editing applications, TALENs are used as sequence-specific DNA-binding domains engineered to direct the nonspecific DNA cleavage domains (nuclease) to the target locus. Similarly, FokI endonuclease can be engineered to form a complex with TALE proteins to create a DSB at the target sequence. Again, like the ZFNs, FokI needs to dimerize to effectively create a DSB. Thus, two adjacent TALE binding sites at opposite strands that are separated by a spacer sequence of 12-20 bp are required to create a targeted cleavage (Figure 3).

CRISPR/Cas9 System

The latest achievement in the field of genome engineering is the "Clustered, Regularly-Interspaced, Short Palindromic Repeats (CRISPR)–Associated protein system (/Cas). CRISPR's fundamentals ile in prokaryotes in the provision of adaptive immunity against viruses and plasmids [42]. The S. pyogenes SF370 Type II CRISPR locus contains four genes: Cas9 nuclease; two noncoding CRISPR RNAs (crRNAs); trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) and nuclease guide sequences (spacers) interspaced by identical direct repeats [43]. The primary function of the RNA components is to direct the Cas9 endonuclease to the specific DNA sequences in order to generate DSBs in these foreign genetic sequences (thus protecting prokaryotic host cells from infection). This adaptive immune system is essential to bacteria and archaea for protection against invading organisms. CRISPRs were initially identified in the E. coli genome in 1987. It was found that E. coli genome contains a series of 29 nucleotide repeats that are separated by unique 32 nucleotide spacer sequences. This repeat-spacer-repeat pattern was later observed in diverse bacterial and archaeal genomes as well. Further studies reveal that these spacer sequences were complementary to various viral and plasmid DNA sequences. This proved that CRISPR was an important adaptive immune element in prokaryotes which also serves as a genetic memory of previous infections [44, 45].

As mentioned above, the S. pyogenes Type II CRISPR system has two varieties of essential components. Out of these, RNA components (crRNA and tracrRNA) merge to form the complex that provides the Cas9 protein with the sequence specificity to cleave the target sequence (which is complementary to the crRNA) [46]. Last year, this natural two-component system was adapted and introduced to eukaryotic organisms such as yeast, plants, and mammals. This novel application was recognized as an easy and effective method of genome

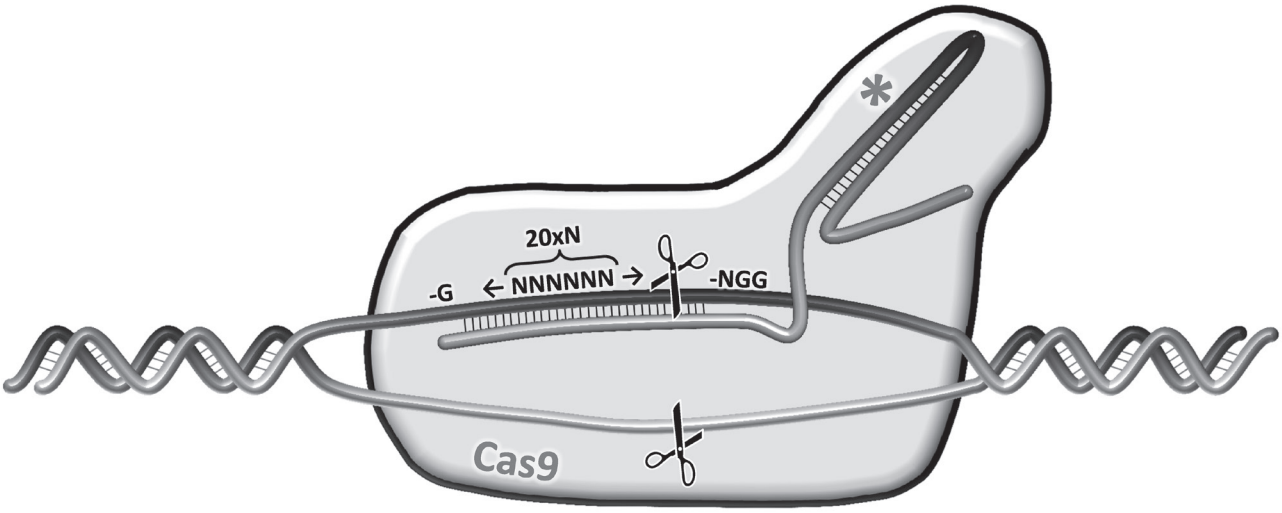

Figure 4. The CrispR/Cas9 system targets the DNA sequence through specific RNA – DNA hybrid helix binding. The RNA element of the system exerts a dual action. Primarily the RNA pairs and binds to the specific locus (through the consensus sequence of G-N20-GG). Once bound to the target site, it also recruits the Cas9 nuclease protein via the structural hairpin loop (labelled with an asterisk). The Cas9 nuclease selectively cleaves both strands at the PAM site (designated as –NGG sequence).

engineering in eukaryotes and enabled efficient genome editing at any target DNA sequence in the mammalian genome. In 2013, Mali et al. modified the protein and RNA components of bacterial Type II CRISPR/Cas system to human cells [47]. Firstly, a human codon-optimized form of the Cas9 protein containing a C-terminal SV40 nuclear localization signal was cloned into a mammalian expression system. The fusion transcript of the RNA components (guide RNAs) was then expressed by the human U6 polymerase III promoter. The guide RNAs are not only necessary but also enable the Cas9 protein to gain sufficient specificity to cleave the target sequence. Mammalian U6 polymerase III promoter transcript starts with a G nucleotide which is required for the PAM (protospacer-adjacentmotif) sequence. The guide RNAs identify this PAM sequence (-NGG) at the target site that harbors the consensus motif of "G-N20-GG" and cut both DNA strands (Figure 4) [47]. Further studies confirm that the Cas9 endonuclease can be directed with a single RNA molecule and provide RNA-programmable genome targeting and editing at any desired sequence [46]. Additionally, a single CRISPR system enables simultaneous modification of various sites within the mammalian genome. However, efficiency of the RNA-guided nuclease largely depends on the targeted site and the cell type [48].

Implementations for the Human Genetic Diseases

According to the Human Genome Mutation Database, nearly 80% of genetic diseases are caused by single base mutations, substitutions, small deletions, and insertions. Modifying these mutated DNA sequences offers therapeutic benefits for several genetic conditions. Genome editing tools are thus the new hope for future gene correction therapy applications [49]. In order to achieve a precise correction of a mutation or to regulate the expression of desired genes, ZFNs, TALENs and CRISPR/Cas systems are powerful molecular tools that accurately recognize their target within the 3.2 billion base pairs of the human genome [50]. This property of genome editing tools creates a new opportunity to cure hundreds of monogenic diseases. The most recent examples of gene correction applications show that both ZFN and TALEN strategies can be used to correct genetic mutation that is responsible for sickle cell anemia. The mutation in the human β-globin (HBB) gene could be corrected in patient-derived induced pluripotent stem cells (hiPSCs) [51, 52]. Similarly, disease-causing mutations in the Parkinson's disease-associated alpha-synuclein (SNCA) gene were also corrected in hiPS cells that have been generated from fibroblasts using ZFNs [53]. In 2011, the biallelic correction of

a point mutation (Glu342Lys) in the α1-antitrypsin (A1AT) gene causing α1-antitrypsin deficiency was achieved [54]. It has also been shown that ZFNs can efficiently correct the X-SCID mutation in the IL2Rr gene locus in both transformed human cells and primary T-cells [55]. These studies are also important for autologous transplantation strategies in which patient-derived cells might be corrected ex vivo and then reintroduced into donors, with reduced risk of immune response [56]. Moreover, it was shown that targeted genome editing could achieve reading frame correction in the dystrophin gene and restore the dystrophin expression in the cells from DMD patients, including skeletal myoblasts and dermal fibroblasts [57]. Genome editing with the CRISPR/Cas9 system was utilized to disrupt the Pcsk9 gene in vivo with high efficiency, and off-target mutagenesis was not detected in ten selected sites at the genome. Pcsk9 inactivation has aided in the reduction of the low-density lipoprotein cholesterol (LDL-C) levels in mice. This approach thus provides remarkable protection against susceptibility for cardiovascular diseases, with a future therapeutic potential in humans [58]. Yet another study shows that mice carrying dominant mutations in the cataract-causing Crygc gene could be corrected by the CRISPR/Cas system. Here, the gene correction was achieved via the HR mechanism, with very rare evidence of off-target mutations. Further studies reveal that the hereditary correction could be achieved at the target locus and resulting mice could transmit the corrected allele to their progeny [59]. Lastly, the CRISPR/Cas9 genome editing system was successfully used to correct the CFTR locus by HR in cultured intestinal stem cells of Cystic Fibrosis patients. The corrected allele was expressed and shown to be functional in expanded organoids. This study also proves that targeted gene correction is possible by HR in primary adult stem cells (somatic stem cells) derived from patients with hereditary single gene defects [60].

In addition to the above-mentioned examples of gene correction, genes may also be disrupted by genome editing to generate therapeutic phenotypes. For example, the HIV co-receptor CCR5 was disrupted in T-cells to block HIV entry [36]. CCR5 was also previously targeted in human hematopoietic stem cells by zinc-finger nucleases to limit HIV infection [37]. This CCR5 targeting approach is currently in Phase 2 clinical trial for the treatment of HIV/AIDS as a cell-based therapy. Another study targets the glucocorticoid receptor gene by ZFNs in CD8+ cytotoxic T lymphocytes (CTL) as part of a T cell based cancer immunotherapy. These modified T-cells have the ability to destroy glioblastoma tumor cells in animals in the presence of glucocorticoids. This initiation is currently in Phase 1 clinical trial for the evaluation of safety and tolerability of engineered T-cells (NCT01082926) [61, 62]. A recent study also shows that TALENs provide a potential for the treatment of chronic hepatitis B (HBV) infection [63]. The functionality of the episomal covalently-closed circular HBV DNA (cccDNA) and suppressed viral replication markers could be destroyed in both cultured cells and in vivo. This is a considerable advance in the therapeutic application of TALENs. Gene inactivation strategies can also be used to generate disease models in model organisms. TALENs have been used to inactivate the gene encoding low-density lipoprotein (LDL) receptor in pigs, thereby generating a model for familial hypercholesterolemia [64]. Rats with X-Linked Severe Combined Immunodeficiency (XSCID) were generated with ZFNs targeting the rat interleukin 2 receptor gamma (Il2rg) locus [65]. Moreover, the renin gene was targeted via ZFNs to create renin knockout rats for cardiovascular disease models [66]. Recent studies demonstrate that CRISPR/Cas systems can efficiently and simultaneously generate single- and multiple-gene mutations in rats [67].

Further studies show that, in addition to single gene disorders, other complex genetic diseases, such as cancer, can potentially be treated by the activation of target genes. For example, the tumor suppressor maspin (SERPINB5) is epigenetically silenced in several types of epithelial tumors. Reactivation of maspin with zinc-finger transcription factors prevents tumor cell metastasis in vitro and tumor xenograft growth in vivo [68]. In addition to the activation of tumor suppressors, oncogene inactivation could also be accomplished via ZFN technology. Falke et al. showed that activation of the expression of the proapoptotic factor Bax by zinc-finger transcription factors induces cell death selectively in cancer cells through the inactivation of the tumor suppressor p53 [69]. Lastly, BAK and BAX deletion by zinc-finger nucleases generated apoptosis-resistant mammalian cell lines (CHO cells) for the production of improved biopharmaceutical compounds [70]. Large scale and high-throughput gene disruptions via the CRISPR/Cas system can greatly

facilitate target identification and drug discovery studies for pharmaceutical uses [31].

A Comparative Analysis of Genome Editing Technologies

The above-summarized examples show that all three genome editing technologies (ZFN, TALEN, and CRISPR/Cas system) can be used for gene disruption, gene correction, and genome engineering in a variety of host cells and organisms, including mammals and humans. However, the efficiency and specificity of genome targeting depends on the locus targeted, the cell type utilized and the properties of the model organism chosen [31]. Genome-wide analysis of ZFN specificity reveals that a low but reckonable rate of off-target effect is present [71]. It has also been shown that ZFNs contain profound cytotoxicity and off-target effects compared to TALENs [72]. In one comparative study, TALENs were designed to disrupt the human CCR5 receptor locus, and successful editing was achieved in up to 45% of transfected cells. A similar gene disruption activity was also achieved by ZFNs in a side-by-side comparison. TALENs, however, exhibited a significantly reduced cytotoxicity. Moreover, the CCR5-specific TALENs revealed minimal off-target activity at the CCR2 locus compared to the corresponding ZFNs (CCR5 and CCR2 exhibit high sequence homology). Principally higher cytotoxic activity and off-target effects as well as the complex design process of ZFNs are the major drawbacks of this system. The labor-intensive requirements to reengineer and confirm the activity of the two opposite strand ZF motifs as well as optimizing the linker spacers render ZFNs relatively difficult to optimize. Thus, despite ongoing clinical trials and the positive progress of TALENs, the final "boom" of the CRISPR/Cas9 system surpassed the ZFNs for targeted modifications of complex genomes.

TALEN technology provides opportunities for targeted gene replacement or mutations at the genomic sequence of interest. Compared to ZNFs, TALENs provide higher targeting flexibility, efficiency and simplicity. The frequent off-target cleavage is the primary cause of the cytotoxic effects of the ZFNs. It is due to the fact that TALENs exhibit higher sensitivity to mismatches that they do not harbor any such drawbacks. Moreover, studies show that three to four mismatches in the target recognition site typically block the DNA-binding capacity of the TAL effectors [73].

The final achievement in genome editing is the CRISPR/Cas system. Several studies show a much higher target specificity of the CRISPR/Cas system compared to both ZFNs and TALENs. Moreover, adverse off-target risks of the RNA-guided mechanism are much lower than those of other genome editing technologies. Cong et al. showed that a single-base mismatch up to 11 bp 5' of the PAM completely blocks the action of Cas9 nuclease. However, mutations further upstream of the spacer may not diminish the nuclease activity [48]. In contrast to ZFPs and TALENs, which require the design and verification of a new engineered protein for each target, the CRISPR system only needs to change the 20 base pair protospacer sequence within the CRISPR expression vector to target a new site. Thus, RNA-programmed Cas9 offers an easy opportunity for gene targeting and genome editing applications [46]. The specificity of RNA-guided endonucleases can be established relatively easily by the design of guide RNA molecules complementary to the target sequence so as to achieve Cas9-mediated DSBs at the desired locus.

One other diversion of the CRISPR/Cas system from ZFNs or TALENs is the mechanism of nuclease action that drives DSBs. In CRISPR/Cas, DSBs are generated through the Cas9 nuclease without any need for the generation or optimization of a chimeric FokI restriction endonuclease. This is one of the advantages of the CRISPR/Cas system. Following the generation of DSBs at the genome, the common endogenous repair mechanisms of the target cell are activated. The choice of repair process is independent of the genome editing tool used for the induction of DSBs. Either an error-prone NHEJ is activated or, as mentioned above, HR may be used to introduce any exogenous homologous sequence within the target locus in the presence of a similar DNA sequence. Consequently, it has been shown that by the use of the CRISPR/Cas system, efficient and simple genome editing is possible. Further improvements to the CRISPR/Cas system are also required to increase its efficiency. Some limitations of this system include the requirement of the consensus motifs for the Cas nuclease activity or the attainability of the target site due to chromatin and DNA methylation states. However, other diverse members of the Cas9 family with various PAM sequences can be readily introduced to overcome these obstacles [48]. Moreover, in a recent study, Veres et al. showed off-target mutagenic effects of these

systems to be extremely rare [74]. By using CRISPR/Cas and TALEN-targeted human pluripotent stem cell clones, they performed whole-genome sequencing at high coverage to determine the degree of mutagenesis in the entire genome. This genome-wide analysis study shows that although off-target mutations may be a risk for some cell types, the probability of such effects in human pluripotent stem cells may be adequately low and not a significant concern for disease modeling and similar applications.

Outlook

The examples summarized above show that genome editing tools are the next novel therapeutic tools for the advancement of human health. Besides their current widely accepted use for the creation of cell- or animal-based genetic models, further clinical trials are also impending, targeting several single gene disorders. Although future applications towards the cure of infectious diseases and cancer have been initiated, we are still far from any efforts targeting common diseases. The major reason for this is the fact that a multitude of alleles with low penetrance but relatively high frequency are the principal causes of these pathologies. Our current level of knowledge is evidently still far from understanding the genetic fundamentals of these multi-component variations. A careful look at the clinical trials database deduces that the majority of the on-going trials are cancer gene therapy trials. Somatic cell therapy targeting cancer cells is a major challenge in itself, with multiple hurdles. Finding voluntary patients to be enrolled in these trials, however, is not an obstacle. On the other hand, our current understanding of the risks of the off-target effects of the genome editing tools is a major drawback for any implementations on children with single gene disorders. The unfortunate consequences of previous trials with retroviruses necessitate further precautions to be taken with such approaches.

At the forefront of this new genome engineering revolution, the authors have initiated the primary research project at Hacettepe University in 2013 using the CRISPR/Cas system to introduce targeted mutations in stem cell lines. Once proven to be beneficial, this approach can be implemented on embryonic stem cells for the creation of animal models as well. The current perspective on genome engineering deduces that the future directions are likely to provide specific somatic gene correction therapy opportunities for several conditions that are currently accepted as "intractable" for clinicians.

REFERENCES

[1] **Avery OT, Macleod CM, McCarty M.** Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types: Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. J Exp Med 1944; 79: 137-158.

[2] **Min Jou W, Haegeman G, Ysebaert M, Fiers W.** Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein. Nature 1972; 237: 82-88.

[3] **Pai GS, Sprenkle JA, Do TT, Mareni CE, Migeon BR.** Localization of loci for hypoxanthine phosphoribosyltransferase and glucose-6-phosphate dehydrogenase and biochemical evidence of nonrandom X chromosome expression from studies of a human X-autosome translocation. Proc Natl Acad Sci U S A 1980; 77: 2810-2813.

[4] **Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A,** *et al.* A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature 1996; 380: 152-154.

[5] **Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, Baehner RL,** *et al.* Cloning the gene for an inherited human disorder—chronic granulomatous disease—on the basis of its chromosomal location. Nature 1986; 322: 32-38.

[6] **International Human Genome Sequencing C.** Finishing the euchromatic sequence of the human genome. Nature 2004; 431: 931-945.

[7] **Hacein-Bey-Abina S, Hauer J, Lim A, Picard C, Wang GP, Berry CC,** *et al.* Efficacy of gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 2010; 363: 355-364.

[8] **Lu QL, Yokota T, Takeda S, Garcia L, Muntoni F, Partridge T.** The status of exon skipping as a therapeutic approach to duchenne muscular dystrophy. Mol Ther 2011; 19: 9-15.

[9] **Plautz GE, Nabel EG, Fox B, Yang ZY, Jaffe M, Gordon D,** *et al.* Direct gene transfer for the understanding and treatment of human disease. Ann N Y Acad Sci 1994; 716: 144-153.

[10] **Hojman P, Gissel H, Andre FM, Cournil-Henrionnet C, Eriksen J, Gehl J,** *et al.* Physiological effects of high- and low-voltage pulse combinations for gene electrotransfer in muscle. Hum Gene Ther 2008; 19: 1249-1260.

[11] **Beutler E.** The Cline affair. Mol Ther 2001; 4: 396-397.

[12] **Naldini L, Blomer U, Gage FH, Trono D, Verma IM.** Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci U S A 1996; 93: 11382-11388.

[13] **Szybalska EH, Szybalski W.** Genetics of human cess line. IV. DNA-mediated heritable transformation of a biochemical trait. Proc Natl Acad Sci U S A 1962; 48: 2026-2034.

[14] **Lesch M, Nyhan WL.** A Familial Disorder of Uric Acid Metabolism and Central Nervous System Function. Am J Med 1964; 36: 561-570.

[15] **Temin HM.** Mixed infection with two types of Rous sarcoma virus. Virology 1961; 13: 158-163.

[16] **Rogers S, Lowenthal A, Terheggen HG, Columbo JP.** Induction of arginase activity with the Shope papilloma virus in tissue culture cells from an argininemic patient. J Exp Med 1973; 137: 1091-1096.

[17] **Terheggen HG, Lowenthal A, Lavinha F, Colombo JP, Rogers S.** Unsuccessful trial of gene replacement in arginase deficiency. Z Kinderheilkd 1975; 119: 1-3.

[18] **Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M,** *et al.* T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science 1995; 270: 475-480.

[19] **Romero NB, Braun S, Benveniste O, Leturcq F, Hogrel JY, Morris GE,** *et al.* Phase I study of dystrophin plasmid-based gene therapy in Duchenne/Becker muscular dystrophy. Hum Gene Ther 2004; 15: 1065-1076.

[20] **Wilson JM.** Medicine. A history lesson for stem cells. Science 2009; 324: 727-728.

[21] **Wirth T, Parker N, Yla-Herttuala S.** History of gene therapy. Gene 2013; 525: 162-169.

[22] **Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F,** *et al.* Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet 2009; 374: 1597-1605.

[23] **Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F,** *et al.* Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 2010; 467: 318-322.

[24] **Aiuti A, Cattaneo F, Galimberti S, Benninghoff U, Cassani B, Callegaro L,** *et al.* Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N Engl J Med 2009; 360: 447-458.

[25] **Boztug K, Schmidt M, Schwarzer A, Banerjee PP, Diez IA, Dewey RA,** *et al.* Stem-cell gene therapy for the Wiskott-Aldrich syndrome. N Engl J Med 2010; 363: 1918-1927.

[26] **Bryant LM, Christopher DM, Giles AR, Hinderer C, Rodriguez JL, Smith JB,** *et al.* Lessons learned from the clinical development and market authorization of Glybera. Hum Gene Ther Clin Dev 2013; 24: 55-64.

[27] **Ferreira V, Twisk J, Kwikkers K, Aronica E, Brisson D, Methot J,** *et al.* Immune responses to intramuscular administration of alipogene tiparvovec (AAV1-LPL (S447X)) in a phase II clinical trial of lipoprotein lipase deficiency gene therapy. Hum Gene Ther 2014; 25: 180-188.

[28] **Carroll D.** Genome engineering with zinc-finger nucleases. Genetics 2011; 188: 773-782.

[29] **Capecchi MR.** Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 2005; 6: 507-512.

[30] **Gaj T, Gersbach CA, Barbas CF, 3rd.** ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 2013; 31: 397-405.

[31] **Cai M, Yang Y.** Targeted genome editing tools for disease modeling and gene therapy. Curr Gene Ther 2014; 14: 2-9.

[32] **Niu J, Zhang B, Chen H.** Applications of TALENs and CRISPR/Cas9 in Human Cells and Their Potentials for Gene Therapy. Mol Biotechnol 2014; 56: 681-688.

[33] **Rouet P, Smih F, Jasin M.** Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol Cell Biol 1994; 14: 8096-8106.

[34] **Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, Kim YG,** *et al.* Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. Mol Cell Biol 2001; 21: 289-297.

[35] **Deng SS, Wang YZ, Ma D.** [Zinc finger nucleases and their application]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2010; 27: 162-165.

[36] **Perez EE, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O,** *et al.* Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 2008; 26: 808-816.

[37] **Holt N, Wang J, Kim K, Friedman G, Wang X, Taupin V,** *et al.* Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat Biotechnol 2010; 28: 839-847.

[38] **Scholze H, Boch J.** TAL effectors are remote controls for gene activation. Curr Opin Microbiol 2011; 14: 47-53.

[39] **Boch J, Bonas U.** Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol 2010; 48: 419-436.

[40] **[40] Tong C, Huang G, Ashton C, Wu H, Yan H, Ying QL.** Rapid and cost-effective gene targeting in rat embryonic stem cells by TALENs. J Genet Genomics 2012; 39: 275-280.

[41] **Li T, Huang S, Zhao X, Wright DA, Carpenter S, Spalding MH,** *et al.* Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res 2011; 39: 6315-6325.

[42] **Wiedenheft B, Sternberg SH, Doudna JA.** RNA-guided genetic silencing systems in bacteria and archaea. Nature 2012; 482: 331-338.

[43] **Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA,** *et al.* CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 2011; 471: 602-607.

[44] **Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E.** Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 2005; 60: 174-182.

[45] **Wiedenheft B, Lander GC, Zhou K, Jore MM, Brouns SJ, van der Oost J,** *et al.* Structures of the RNA-guided surveillance complex from a bacterial immune system. Nature 2011; 477: 486-489.

[46] **Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E.** A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012; 337: 816-821.

[47] **Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE,** *et al.* RNA-guided human genome engineering via Cas9. Science 2013; 339: 823-826.

[48] **Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N,** *et al.* Multiplex genome engineering using CRISPR/Cas systems. Science 2013; 339: 819-823.

[49] **Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L,** *et al.* Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 2014; 156: 836-843.

[50] **Gersbach CA, Perez-Pinera P.** Activating human genes with zinc finger proteins, transcription activator-like effectors and CRISPR/Cas9 for gene therapy and regenerative medicine. Expert Opin Ther Targets 2014; 18: 835-839.

[51] **Sebastiano V, Maeder ML, Angstman JF, Haddad B, Khayter C, Yeo DT,** *et al.* In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. Stem Cells 2011; 29: 1717-1726.

[52] **Sun N, Liang J, Abil Z, Zhao H.** Optimized TAL effector nucleases (TALENs) for use in treatment of sickle cell disease. Mol Biosyst 2012; 8: 1255-1263.

[53] **Soldner F, Laganiere J, Cheng AW, Hockemeyer D, Gao Q, Alagappan R,** *et al.* Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell 2011; 146: 318-331.

[54] **Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, Paschon DE,** *et al.* Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. Nature 2011; 478: 391-394.

[55] **Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S,** *et al.* Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 2005; 435: 646-651.

[56] **Joung JK, Sander JD.** TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 2013; 14: 49-55.

[57] **Ousterout DG, Perez-Pinera P, Thakore PI, Kabadi AM, Brown MT, Qin X,** *et al.* Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. Mol Ther 2013; 21: 1718-1726.

[58] **Ding Q, Strong A, Patel KM, Ng SL, Gosis BS, Regan SN,** *et al.* Permanent Alteration of PCSK9 With In Vivo CRISPR-Cas9 Genome Editing. Circ Res 2014.

[59] **Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S,** *et al.* Correction of a genetic disease in mouse via use of CRISPR-Cas9. Cell Stem Cell 2013; 13: 659-662.

[60] **Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T,** *et al.* Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 2013; 13: 653-658.

[61] **Torikai H, Reik A, Soldner F, Warren EH, Yuen C, Zhou Y,** *et al.* Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. Blood 2013; 122: 1341-1349.

[62] **Reik A, Zhou Y, Collingwood TN, Warfe L, Bartsevich V, Kong Y,** *et al.* Enhanced protein production by engineered zinc finger proteins. Biotechnol Bioeng 2007; 97: 1180-1189.

[63] **Bloom K, Ely A, Mussolino C, Cathomen T, Arbuthnot P.** Inactivation of hepatitis B virus replication in cultured cells and in vivo with engineered transcription activator-like effector nucleases. Mol Ther 2013; 21: 1889-1897.

[64] **Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M,** *et al.* Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci U S A 2012; 109: 17382-17387.

[65] **Mashimo T, Takizawa A, Voigt B, Yoshimi K, Hiai H, Kuramoto T,** *et al.* Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. PLoS One 2010; 5: e8870.

[66] **Moreno C, Hoffman M, Stodola TJ, Didier DN, Lazar J, Geurts AM,** *et al.* Creation and characterization of a renin knockout rat. Hypertension 2011; 57: 614-619.

[67] **Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F,** *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 2013; 153: 910-918.

[68] **Beltran A, Parikh S, Liu Y, Cuevas BD, Johnson GL, Futscher BW,** *et al.* Re-activation of a dormant tumor suppressor gene maspin by designed transcription factors. Oncogene 2007; 26: 2791-2798.

[69] **Herrmann F, Garriga-Canut M, Baumstark R, Fajardo-Sanchez E, Cotterell J, Minoche A,** *et al.* p53 Gene repair with zinc finger nucleases optimised by yeast 1-hybrid and validated by Solexa sequencing. PLoS One 2011; 6: e20913.

[70] **Cost GJ, Freyvert Y, Vafiadis A, Santiago Y, Miller JC, Rebar E,** *et al.* BAK and BAX deletion using zinc-finger nucleases yields apoptosis-resistant CHO cells. Biotechnol Bioeng 2010; 105: 330-340.

[71] **Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, Kaeppel C,** *et al.* An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 2011; 29: 816-823.

[72] **Mussolino C, Morbitzer R, Lutge F, Dannemann N, Lahaye T, Cathomen T.** A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. Nucleic Acids Res 2011; 39: 9283-9293.

[73] **Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S,** *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. Science 2009; 326: 1509-1512.

[74] **Veres A, Gosis BS, Ding Q, Collins R, Ragavendran A, Brand H,** *et al.* Low Incidence of Off-Target Mutations in Individual CRISPR-Cas9 and TALEN Targeted Human Stem Cell Clones Detected by Whole-Genome Sequencing. Cell Stem Cell 2014; 15: 27-30.